2.3 Biological activities

Rocaglamide and the analogues **2–13** have been tested against the following insects:

Heliothis virescens F
- Hel.e/l egg-larval on artificial diet
- Hel.L1 first instar on soybean
- Hel.L3 third instar on soybean

Spodoptera littoralis Boisd
- Spo.L3 third instar on soybean

Plutella xylostella L
- Plu.L2/3 second/third instar on cabbage

Diabrotica balteata Lec
- Dia.L2 second instar on maize seedlings

The compounds were tested either as a 500 g litre^{-1} emulsifiable concentrate or in acetone + water solutions.

3 RESULTS

3.1 Structure–activity relationship

The compounds **1** to **5**, bearing the cyclopentatetrahydrobenzofuran ring (Fig 1) show potent insecticidal activities, rocaglamide being the most active (Table 1).

This cyclopentatetrahydrobenzofuran moiety is also present in the pyrimidinone analogues **7** to **10**, (Fig 2). However, due to the planar pyrimidinone ring, these compounds display a completely different steric configuration, but are still active.

The cyclopentatetrahydrobenzopyran derivatives **11** to **16** (Fig 3) are inactive. They may be formed by addition of a flavone (kaempferol?) and odorine, a natural diamine also found in *Aglaia* species.

4 CONCLUSION

We have isolated 15 new analogues of rocaglamide. Rocaglamide and the furan analogues **2** to **5** are the most active compounds isolated. However, the insecticidal activity of rocaglamide could not be improved upon.

Meaningful information on structure–activity relationships can be uncovered by the detailed study of the complete spectrum of natural products occurring in biologically active extracts.

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Bioactive compounds from neem tissue cultures and screening against insects

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Abstract: Hairy root cultures have been derived from neem (*Azadirachta indica* A Juss, Family Meliacceae) using *Agrobacterium rhizogenes* and

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have been studied for the production of compounds with antifeedant effects on insects. Six-week-old hairy root cultures were extracted, and HPLC yielded fractions ranging from polar to non-polar compounds. High antifeedancy levels against the desert locust were observed in fractions(F) 2, 3 and 4 whilst F1 and F5 were not significantly antifeedant. Interestingly F3 did not contain any of the well-known neem chemicals while F2 contained azadirachtin and 3-tigloylazadirachtol and F4 nimbin and salannin.

Keywords: neem; hairy roots; azadirachtin; antifeedancy

1 INTRODUCTION

Extracts from the neem tree (*Azadirachta indica* A Juss, Family, Meliacceae) are widely exploited for their use against insects. In particular the complex neem triterpenoid azadirachtin has been studied intensively and found to be an insect antifeedant, growth regulator (IGR) and sterilant.[1,2] However, azadirachtin has proved to be too complex to produce synthetically on a commercial basis.[3] Thus, the supply of neem-based insecticides depends upon the extraction of seeds, which are difficult to obtain in the quantity and quality necessary to ensure high azadirachtin content.

Neem tissue cultures have been developed and studied as an alternative method for product synthesis. Although undifferentiated neem cell and callus cultures have been reported to produce azadirachtin,[4–6] differentiated cultures possess advantages such as higher genetic stability and greater production of secondary metabolites with time.[7] To this end, differentiated neem hairy root cultures have been established and studied. The aim of this study was to investigate and determine the capacity of a neem hairy root line for production of azadirachtin and other biochemicals. This was carried out by chemical analysis of the root extracts, and the biological activity of the extracts was monitored with feeding bioassays against the desert locust [*Schistocerca gregaria* (Forskål)] in a 'choice' situation.

2 EXPERIMENTAL

2.1 Growth and harvest of hairy root cultures

A hairy root line (derived from leaf explants from identified neem trees originating in the dry zone of Sri Lanka near the village of Dambulla) was used in this study.[8] Cultures were grown in full strength, hormone-free Murashige and Skoog basal medium[9] supplemented with sucrose (30 g litre^{-1}; M&S). The pH was adjusted to 5.8. Cultures were maintained by routine subculture at six-week intervals in 250 ml flasks containing M&S (100 ml) inoculated with 30 root tips of 1–2 cm in length (approx. 0.1 g fresh weight). Cultures were grown in an orbital shaker (Gallenkamp, UK) at 90 rev min^{-1} in the dark at 25°C.

Hairy roots were harvested from their late exponential growth phase (six weeks of culture) under vacuum through Mira cloth (Calbiochem Corporation, USA). Immediately after harvest the roots were flash frozen with liquid nitrogen, freeze dried (Edwards, UK) and stored at −20°C prior to extraction.

2.2 Extraction of hairy roots

Freeze dried hairy roots (1 g dry weight) were flash frozen in liquid nitrogen, ground manually and homogenized (Intern. App. GmbH) with methanol (30 ml) for 10 min. The meal was then filtered through Whatman No 1 filter paper under vacuum and the solids rinsed from the filter paper with a further 30 ml of methanol and homogenized for another 10 min. Methanol extract + distilled water (2 : 1 by volume; 100 ml) was shaken with dichloromethane (60 ml) and the resultant meal allowed to settle for 10–15 min. The dichloromethane layer was separated, dried over anhydrous magnesium sulfate and filtered as above. The extract was evaporated to dryness in a rotary evaporator at 18–20°C and the residue resuspended in dichloromethane.

2.3 Clean-up of cell extracts

For chromatography, samples were cleaned up by a Solid Phase Extraction (SPE) technique (Morgan DE, pers comm) using pre-packed Bond-elute silica cartridges (1 ml capacity) (Phenomenex, UK). The extraction procedure involved a system of solvents of increasing polarity, obtained by mixing the non-polar light petroleum (bp 60–80°C) with an increasing preposition of the polar ethyl acetate. After washing, extracts were combined, evaporated to dryness and resuspended in acetonitrile (HPLC grade) + water (10 : 90 by volume) for HPLC analysis.

2.4 High performance liquid chromatography (HPLC)

The HPLC system consisted of two 302 pumps, a 20 µl loop injector, a 116 UV detector and a Gilson 715 system controller and data processor. A primesphere C18-MC column (5 µm particle size, 25 cm length, Phenomenex, UK) with a guard column (5 cm) was used and detection was carried out at a wavelength of 217 nm. The sensitivity was 0.001 AU. The HPLC method used initially was based on the method of Eeswara[8] with modified mobile phase gradient and a flow rate of 1 ml min^{-1} (Table 1). The chromatographic run time was 45 min. Fractions eluting at 7.01–12.01 (F1), 12.02–16.74 (F2), 16.75–21.99 (F3), 22.00–26.79 (F4) and 26.80–32.00 (F5) min after injection were selected for biological testing.

2.5 Choice feeding insect bioassay

The choice feeding bioassay is based on the method of Mordue(Luntz) *et al.*[5] Male and female three- to

Table 1. HPLC solvent gradient systems

Time (min)	*Acetonitrile (% v/v)*
0	10
10	10
12	40
17	40
35	70
37	100
39	100
41	10
45	10

four-day-old desert locust (*Schistocerca gregaria*. Forskål) fifth-instar nymphs were used in the bioassays. Insects were starved overnight, fed for 1 h on lettuce and starved again for 3 h to standardize their state of hunger. At the end of the second period of food deprivation, each insect was individually transferred into a container and was given across to two glass-fibre discs 3.5 cm diameter (Whatman International Ltd, UK). These had been pretreated with sucrose (50 mM; 350 µl) and allowed to oven dry at 37°C overnight, after which one received 350 µl of an appropriate extract in ethanol and the other ethanol alone (control); the discs were then dried again under the same conditions. All discs were weighed before and after the experiment, the difference being recorded as mg disc eaten. The percentage of antifeedancy was calculated as:

$$\frac{\text{Weight of control disc eaten} - \text{Weight of test disc eaten}}{\text{Weight of control disc eaten} + \text{Weight of test disc eaten}} \times 100$$

Results were analysed using one-way analysis of variance after arcsin transformation and means were compared by Duncan's multiple range test (SPSS statistic package). ED_{50} values were calculated after probit analysis.

Table 2. Effects of fractions from hairy root cultures of *Azadirachta indica* on food intake by fifth-instar nymphs of *Schistocerca gregaria* in a choice feeding bioassay[a]

Fractions	*Antifeedancy* (%) $\pm$(*SE*)[b]
1	0
2	62.2($\pm$9.8)a
3	56.1($\pm$6.5)a
4	23.9($\pm$7.7)b
5	0

[a] Tested at 0.1 mg ml^{-1}; n = 7–12.
[b] Means followed by the same letter are not significant different, $P \leqslant 0.05$; Duncan's multiple range test.

3 RESULTS AND DISCUSSION

3.1 HPLC analysis

The reverse-phase HPLC retention times for azadirachtin, 3-tigloylazadirachtol, nimbin and salannin were 15.03, 15.1, 25.3 and 26.2 min, respectively. The presence of the above compounds in the extracts was tested by co-injection of the extracts with samples of pure compounds. Fraction F2 contained azadirachtin and 3-tigloylazadirachtol and F4 contained nimbin and salannin. Further confirmation of the identity of the compounds is being carried out using isocratic methodologies to separate azadirachtin and 3-tigloylazadirachtol.

3.2 Bioactivity of the hairy root extracts

S gregaria is particularly sensitive to azadirachtin[2] and to neem extracts,[5–10] considering them as a mixture of compounds, at the behavioural level. Thus, the use of that species in the present work has allowed the comparison of the potential of different neem hairy root fractions as antifeedants (Table 2). The five fractions were tested in a range of concentrations from 0.05 to 10.0 mg dry weight of plant material per ml. Fraction F2 gave 62% antifeedancy at 0.1 mg ml^{-1}, but only F3 showed similar activity. F4, which contained nimbin and salannin, produced 23% antifeedancy, which was significantly different from F2 and F3. F1 and F5 were active only at concentrations (10 mg ml^{-1}) 100 times higher, indicating that there were no compounds with significant antifeedant activity present in these fractions. Undifferentiated neem cultures have also been reported to produce antifeedant compounds, in addition to azadirachtin, which have not yet been identified,[4] but these are known to be less polar than azadirachtin and more polar than nimbin and salannin. The fractions F2, F3 and F4 gave good dose-response antifeedancy relationships, with ED_{50} values of 0.098, 0.126 and 0.427 mg ml^{-1}, respectively.

Future work will concentrate on isolation and identification of these compounds. Also, the enhancement of product yields in terms of azadirachtin and other biochemicals which are produced from the cultures will be studied.

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Transport of [^{14}C]benazolin and bromide in large zero-tension outdoor lysimeters and the undisturbed field in a sandy soil

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Abstract: In order to investigate the transferability of lysimeter results to the actual field situation, a leaching study with [^{14}C]benazolin and bromide was carried out in a sandy soil. A suction base system, where soil water and solute fluxes through a lateral cross-sectional sampling area could be measured in an undisturbed field environment, was developed as reference system. Using that measuring instrument, possible artefacts of the lysimeter system could be excluded. The outflow of soil water and leaching of benazolin and bromide showed no system-related differences between the lysimeters and the undisturbed field represented by the suction base station. Higher outflow of leachate and bromide in the lysimeters could be attributable to different meteorological conditions at the lysimeter and the field station.

Keywords: lysimeter; transferability; suction base; solute transport; benazolin; sandy soil

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Pesticide leaching studies with large suction-free lysimeters are an important approach to assess the leaching behaviour of pesticides. In contrast to field studies, where only a small fraction of the entire transport system can be sampled, they provide for a complete detection of solutes that reach the sampling depth. Additionally, ^{14}C-labelled pesticides can be used with all the known advantages of the tracer technique.

However, differences between zero-tension lysimeters and the undisturbed field may affect pesticide transport and limit the transferability of the results. In particular the capillary fringe at the lysimeter ground, which is caused by the zero-tension conditions at the lower boundary, may artificially influence the soil water and solute dynamics. In addition, cracks and fissures, which may result from sampling and transport of the monoliths, may induce artificial flow of solutes. Finally, the lysimeter wall prevents lateral water and solute flux of the solute pulses applied.

In order to compare the leaching behaviour of lysimeters with that of the undisturbed field (loamy sand: 70–78% sand, 15–24% silt, 2–5% clay, <1% C_{org}), three lysimeters were used (0.8 m^2 surface, 1.3 m height), and a research station with three suction bases at a depth of 1.3 m was installed at the field site. The suction bases, which were inserted laterally into the undisturbed soil, consisted of steel frames (1.5 m wide, 1.8 m long, 0.7 m high), which were open at the front and back. Through the perforated ceiling, more than 200 suction cups were inserted into the undisturbed soil above each frame. The potential of the suction cups was permanently adjusted to a relevant value, the mean of several tensiometers at this depth. The system provided an adequate sampling technique for drainage water at a cross-sectional area as in the case of lysimeters. A capillary fringe was avoided, as well as artificial disturbances of the soil above the suction units. Lateral water and solute flow was possible, and thus the artefacts of the lysimeter system could be avoided.[1]

Bromide and [^{14}C]benazolin were applied simultaneously to the six plots of both test systems. The leachate was collected weekly and analysed for bromide concentration and ^{14}C-activity.[1]

After 2.5 years of experimentation and more than 2000 mm precipitation, between 38% and 81% of the bromide and 0.5% and 4.8% of the [^{14}C]benazolin applied was recovered in the effluent (Fig 1). Cumulative leachate and bromide outflow occurred relatively in parallel, revealing a CV (Coefficient of Variation) of 9% and 5% for the lysimeters and 21% and 22% for the suction bases. In contrast, the outflow of [^{14}C]benazolin showed great variability within both systems, resulting in a CV of 94% and 61% for the lysimeters and the suction bases, respectively. However, the mean [^{14}C]benazolin mass in the effluent was similar in both systems, whereas the mean outflows of leachate and bromide were *c*35%